

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>B01D 15/08, C12Q 1/24</b> <b>// G01N 33/569</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 91/16116</b> <b>(43) International Publication Date:</b> 31 October 1991 (31.10.91)
<b>(21) International Application Number:</b> PCT/US91/02780 <b>(22) International Filing Date:</b> 23 April 1991 (23.04.91) <b>(30) Priority data:</b> 513,543 23 April 1990 (23.04.90) US <b>(71) Applicant:</b> CELLPRO INCORPORATED [US/US]; 22322-20th Avenue S.E., Bothell, WA 98021 (US). <b>(72) Inventors:</b> GOFFE, Randal, A. ; 508-225th Place S.E., Bothell, WA 98021 (US). BERENSON, Ronald, J. ; 6127-84th Avenue S.E., Mercer Island, WA 98040 (US). BERNINGER, Ronald, W. ; 10018-64th Place W., Everett, WA 98204 (US). KALAMASZ, Dale ; 12045-184th N.E., Redmont, WA 98052 (US).	<b>(74) Agents:</b> McMASTERS, David, D. et al.; Seed and Berry, 6300 Columbia Center, Seattle, WA 98104-7092 (US). <b>(81) Designated States:</b> AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title:</b> IMMUNOSELECTION DEVICE AND METHOD  <b>(57) Abstract</b>  Devices and methods are provided for selectively isolating or separating target particles from a mixture of target particles and non-target particles. The devices are suitable for the separation of target particles which are indirectly bound to a first member, from a mixture of target particles and non-target particles, comprising: a column having a proximal end with an inlet port through which fluid may exit the column; and a bed of low nonspecific binding porous material within the column, the porous material having a second member capable of binding to the first member with an affinity constant of greater than about $10^8 \text{M}^{-1}$ immobilized on the surface thereof, wherein the pores of the porous material are of a size sufficient to allow the second member to enter into the pores and to allow entry of molecules greater than about 35 kD, but not so large as to allow collapse of the bed, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FK	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

Description

## IMMUNOSELECTION DEVICE AND METHOD

5

Technical Field

The present invention relates to immunoselection devices in general and, more specifically, to devices and methods for selectively isolating or separating target particles from a mixture of target particles and non-target particles.

10

Background of the Invention

Various immunoselection methods exist for separating a particular cell subpopulation, or target cells, from a mixture of cells, as discussed below. Such methods include gross separation with centrifuges, separation by killing of unwanted cells, separation with fluorescence activated cell sorters, separation by directly or indirectly binding cells to a ligand immobilized on a physical support, separation with panning techniques, separation by column immunoadsorption, and separation using magnetic immunobeads. These methods have been used to either positively or negatively select cells. In this regard, positive selection refers to the direct selection and recovery of specific target cells, while negative selection refers to the elimination of a specific target cell subpopulation from a heterogeneous population of cells.

More specifically, centrifuges have been used to separate cells based on differences in cell size and density. However, these devices can only be used to separate cells with large physical differences, such as white blood cells, red blood cells and platelets. They cannot be used where there are minor differences between cells; for example, they cannot be used to separate distinct white blood cell populations.

In addition, a number of techniques have been developed to separate cells by killing unwanted cells. The application of such techniques has been primarily limited to the use of drugs or monoclonal antibodies for killing tumor cells that may be present in autologous marrow prior to transplantation. The drug that is most commonly used is 4-hydroperoxycyclophosphamide. However, clinical studies have indicated that this drug is severely toxic to normal marrow progenitors and that it unpredictably kills tumor cells. Antibodies that react with tumor cells or T cells have also been used to kill unwanted cells. These

antibodies are not capable of killing the cells alone, but require another reagent (complement or toxins) to work in combination with the antibodies to achieve cell death. However, the reactivity of the antibodies with tumor cells is variable and so the killing of tumor cells is often incomplete. The killing agents, such as toxins  
5 or complement, also have adverse effects on normal cellular elements.

As noted above, Fluorescence-Activated Cell Sorters (FACS) have also been used for both negative and positive cell selection. Briefly, a mixture of cells is first incubated with a fluorescent labeled monoclonal antibody that recognizes a particular cell surface antigen. Single cells are then illuminated with  
10 light from a laser and selected on the basis of whether they are stained with the fluorescent antibody. Although the FACS can be effective for separating small numbers of cells for laboratory research, its limited throughput (10 million cells per hour) makes it impractical for separation of the large number of cells required for diagnostic and therapeutic clinical applications. For example, it would take  
15 several hours to separate a 15-20 ml maternal blood sample containing about 20-80 million nucleated cells. The device would take weeks to separate the 10-20 billion marrow cells typically harvested for a transplant. Further, FACS machines are expensive, require highly skilled technicians to operate and are costly to maintain.

20 Recently, two general approaches have been utilized to separate cells which are immobilized on the surface of a physical support or device through the use of monoclonal antibodies (see Berenson et al., PCT Pub. No. WO 87/04628). In one approach, monoclonal antibodies are placed directly on the surface of the cell separation device. Cell selection is achieved by the direct  
25 binding of antigen positive cells to the antibodies immobilized on the surface of the device or support. In the second approach, cells are first incubated with monoclonal antibodies which bind to a surface antigen, and then are exposed to a cell separation device that contains a ligand such as an anti-immunoglobulin antibody, that will bind to antibodies on the cell surface.

30 Within a "panning" technique, cells are separated on a physical support, such as a plastic dish coated with antibodies. More specifically, a mixture of cells is poured onto the coated dish and is incubated so that binding between the antibodies and the target cell surface antigen occurs. After incubation, the dish is washed to remove unbound cells. Panning is simple to use but suffers from  
35 several drawbacks. The bond between antibodies and target cell surface antigens is weak, which means that cells must be incubated for long periods of time on the coated plastic surface for efficient cell binding to occur. During that time, large

numbers of antigen negative cells will also attach to the surfaces resulting in a high degree of nonspecific binding. Furthermore, many antibodies are ineffective because the binding between the antibodies and the cell is too weak for any cellular attachment. The devices are also cumbersome to use for large-scale separation procedures. In addition, red blood cells and nonspecifically bound cells must usually be removed in these procedures before cells can be separated on these devices. Finally, a large amount of antibody must be coated on these surfaces, which makes the devices expensive to produce for clinical applications.

As noted above, magnetic immunobeads have been developed for cell separation, although having been used almost exclusively for negative cell selection. Briefly, cells are first labeled with antibody coated microspheres and then a magnetic device is used to separate labeled from unlabeled cells. The technique has been applied in the clinical setting to deplete tumor cells from the marrow of patients undergoing autologous marrow transplantation. Incubation of cells with these magnetic beads is required for cell binding, resulting in large numbers of cells nonspecifically binding to the magnetic immunobeads. In addition, it is difficult to separate the antigen positive cells from the tiny magnetic immunobeads. These problems have made the technology of limited use for positive cell selection, particularly in the clinical setting.

Column immunoadsorption devices have also been developed for positive as well as negative cell selection. The column typically contains coated beads having a ligand, such as an antibody, immobilized on the surface of the beads. Most of these columns have relied on the relatively weak binding between cell surface antigen and antibody on the beads, and thus require incubation of cells for binding to occur. Indirect immunoadsorption devices have been made that employ ligands such as protein A, plant lectins, fluorescein isothiocyanate, or goat antimouse immunoglobulin. These devices also require incubation in order for cells to bind to the solid surface. Similar to panning and the use of magnetic immunobeads, the incubation of cells with the beads results in a high degree of nonspecific cell binding. Furthermore, many of these ligands can only be used to isolate certain types of cells because of either weak binding or because of the limited range of reactivity of the ligands. In addition, the cost of these ligands has made the scale up to separate the large numbers of cells required for clinical applications impractical.

Recently, an avidin-biotin system has also been used for indirect immunoadsorption. Avidin is a glycoprotein that can be isolated from raw egg whites and has been used as the immobilized ligand. Biotin, a vitamin that is

found in every living cell, binds to avidin with a remarkably high affinity constant of  $10^{15} \text{ M}^{-1}$ . Biotin has been conjugated with antibodies which bind to particular cells or with an anti-immunoglobulin antibody that binds to an antibody bound to a cell surface. In immunoselection methods using biotin-avidin, a mixture  
5 containing cells bound directly to a biotinylated antibody or indirectly to a biotinylated anti-immunoglobulin antibody is brought into contact with immobilized avidin. The target cells are then adsorbed to the immobilized avidin. The depleted mixture may then be recovered or discarded.

Methods such as those described above employ an excess amount of  
10 antibody, biotinylated antibody, or biotinylated anti-immunoglobulin antibody which must be removed before bringing the cells into contact with the immobilized ligand or immobilized avidin. This removal has been required to eliminate free antibody, biotinylated antibody, or biotinylated anti-immunoglobulin antibody that would otherwise compete for the immobilized  
15 ligand or avidin. If excess free antibody, biotinylated antibody, or biotinylated anti-immunoglobulin antibody is not removed, an undesirable amount of target cells may remain in the mixture of cells. This produces either a cell sample still containing a significant amount of the target cells or a population of enriched target cells at a reduced yield.

20 To be clinically useful, cell separation devices and methodologies should be able to effectively isolate target cells from a mixture containing target and non-target cells without significant contamination by non-target cells. It is also critical for the device to have sufficient capacity to process large numbers of cells for most clinical applications. Furthermore, the device should be able to  
25 process cells rapidly and economically, in a simple and readily reproducible manner.

The present invention provides devices and methods that overcome the disadvantages discussed above, while providing other related advantages.

### 30 Summary of the Invention

Briefly stated, the present invention is directed to devices and methods for separating target particles from a mixture of target particles and non-target particles. Within one aspect of the present invention, a device is provided for the separation of target particles bound indirectly to a first member from a  
35 mixture of target particles and non-target particles, comprising (a) a column having a proximal end with an inlet port through which fluid may enter the column and a distal end with an outlet port through which fluid may exit the column; and

(b) a bed of low nonspecific binding porous material within the column, the porous material having a second member capable of binding to the first member with an affinity constant of greater than about  $10^8 \text{ M}^{-1}$  immobilized on the surface thereof, wherein the pores of the porous material are of a size sufficient to allow the second member to enter into the pores, and to allow entry of molecules greater than about 35 kD, but not so large as to allow collapse of the bed, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed. The device may further comprise a means, located within the column, for agitating the porous material upon the application of an external force, such that bound target particles are released from the porous material.

Within a related aspect of the present invention, the bed of low nonspecific binding porous material positioned within the column has a second member capable of binding to the first member with an affinity constant of greater than about  $10^8 \text{ M}^{-1}$  immobilized on the surface thereof. The pores of the porous material are as large as possible without allowing collapse of the bed and of a size sufficient to allow the second member to enter into the pores. In addition, the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed.

Within another related aspect of the invention, the bed of low nonspecific binding porous material comprises porous polyacrylamide beads which are positioned within the column and have a second member capable of binding to the first member with an affinity constant of greater than  $10^8 \text{ M}^{-1}$  immobilized onto the surface thereof. The pores of the beads are of a size sufficient to allow entry of molecules greater than about 60 kD into the pores, but not so large as to allow molecules of greater than about 300 kD into the pores. The interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed. Within this aspect, the device also includes a means for retaining the beads within the column.

Within another aspect of the present invention, a device for the separation of target particles directly or indirectly bound to a biotinylated ligand from a mixture of target particles and non-target particles is provided. The device generally comprises (a) a column having a proximal end with an inlet port through which fluid may enter the column and a distal end with an outlet port through which fluid may exit the column; (b) a bed of low nonspecific binding porous material within the column, the porous material having a biotin adsorbing group immobilized on the surface thereof, wherein the pores of the porous material are

of a size sufficient to allow the biotin adsorbing group to enter into the pores, but not so large as to allow collapse of the bed, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed. The device may further comprise a means, located within the column, for agitating the porous material upon the application of an external force, such that bound target particles are released from the porous material.

Within a related aspect of the present invention, the bed of low nonspecific binding porous material positioned within the column comprises polyacrylamide beads having a biotin adsorbing group immobilized onto the surface thereof, the pores of the beads being of a size sufficient to allow entry of molecules greater than about 60 kD into the pores, but not so large as to allow molecules of greater than about 300 kD into the pores. In addition, the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed.

Within yet another aspect of the invention, a device for the separation of target cells indirectly bound to a first member from a mixture of target cells and non-target cells is provided, comprising (a) a column having a proximal end with an inlet port through which fluid enters the column and a distal end with an outlet port through which fluid exits the column; (b) a bed of low nonspecific binding material within the column, the material having a second member capable of binding to the first member within an affinity constant of greater than about  $10^8 \text{ M}^{-1}$  immobilized on the surface thereof, wherein the interstitial spaces of the bed are of a size sufficient to allow the cells to flow through the bed; and (c) a means for selectively agitating the material upon the application of an external force, such that bound target particles are fractionally released from the material, the agitating means being located within the column.

Within another aspect of the invention, a device for the separation of biotinylated target particles from a mixture of target and non-target particles is provided, comprising (a) a column having a proximal endcap with an inlet port through which fluid may enter the column and a distal endcap with an outlet port through which fluid may exit the column, wherein the endcaps are designed to retain a mesh; (b) a mesh retained in the upper and lower endcaps to retain beads within the column; (c) a bed of low nonspecific binding porous polyacrylamide beads within the column, the beads having a biotin adsorbing group immobilized onto the surface thereof, wherein the pores of the beads are of a size sufficient to allow entry of molecules greater than about 60 kD into the pores, but not so large as to allow molecules of greater than about 300 kD into the pores, and wherein



the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and (d) a magnetic impeller located within the column for agitating the porous polyacrylamide beads upon the application of an external force, such that bound target particles are released from the porous polyacrylamide beads.

Within yet another aspect of the invention, a device for the separation of target particles from a mixture of target particles and non-target particles is provided. The device comprises (a) a column having a proximal end with an inlet port through which fluid may enter the column and a distal end with an outlet port through which fluid may exit the column; (b) a bed of low nonspecific binding porous material within the column, the porous material having a ligand capable of specifically binding to the target particles immobilized on the surface thereof, wherein the pores of the porous material are of a size sufficient to allow the ligand to enter into the pores and to allow entry of molecules greater than about 35 kD, but not so large as to allow collapse of the bed, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed. The device may further comprise a means, located within the column, for agitating the porous material upon the application of an external force, such that bound target particles are released from the porous material.

Within a related aspect of the present invention, the pores of the low nonspecific binding porous material within the column are designed so as to be as large as possible without allowing collapse of the bed and of a size sufficient to allow the ligand to enter into the pores. Within another related aspect of the present invention, the bed of low nonspecific binding porous material comprises porous polyacrylamide beads. The beads have a ligand capable of specifically binding to the target particles immobilized onto the surface thereof, wherein the pores of the beads are of a size sufficient to allow entry of molecules greater than about 60 kD into the pores, but not so large as to allow molecules of greater than about 300 kD into the pores. The interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed. Within this aspect, the device also includes a means for retaining the beads within the column.

Within yet another aspect of the present invention, a sterile system for the separation of target cells from a mixture of target and non-target cells is provided. The system comprises (a) a sterile column that has a proximal end with an inlet port through which fluid may enter the column and a distal end with an outlet port through which fluid may exit the column; (b) a sterile bed of low nonspecific binding porous polyacrylamide beads located within the column

having avidin immobilized on the surface thereof, wherein the pores of the beads are of a size sufficient to allow the entry of molecules of greater than 60 kD into the pores, but not so large as to allow molecules of greater than about 300 kD into the pores, and wherein the interstitial spaces of the bed are of sufficient size to  
5 allow the cells to flow through the bed; (c) a sterile magnetic impeller located within the column; (d) a means for introducing a sterile mixture of cells and other sterile process fluids into the column such that sterility is maintained; (e) a means for collecting cells and process fluids exiting from the column such that sterility is maintained; and (f) an external source of electromagnetic force for regulating the  
10 rotation of the impeller.

As noted above, the present invention also provides methods for separating target particles from a mixture of target particles and non-target particles. In one aspect, the method comprises (a) incubating a mixture of target  
15 particles and non-target particles in the presence of a first member chemically linked to a ligand capable of specifically binding to the target particles under conditions and for a time sufficient to allow binding of the ligand to the target particles; (b) passing the mixture of particles through a bed of low nonspecific binding porous material, the porous material having a second member capable of  
20 binding to the first member with an affinity constant of greater than about  $10^8 \text{ M}^{-1}$  immobilized on the surface thereof, wherein the pores of the porous material are of a size sufficient to allow the second member to enter into the pores and to allow entry of molecules greater than about 35 kD, but not so large as to allow collapse of the bed, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and (c) removing  
25 unbound non-target particles from the column.

Within a related aspect of the present invention, the bed of low nonspecific binding porous material has a second member capable of binding to the first member with an affinity constant of greater than about  $10^8 \text{ M}^{-1}$  immobilized onto the surface thereof, the pores of the porous material being as  
30 large as possible without allowing collapse of the bed and of a size sufficient to allow the second member to enter into the pores. Further, the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed. Within another related aspect of the present invention, the bed of low nonspecific binding porous material is a bed of porous polyacrylamide beads, as described  
35 above.

Within yet another aspect of the present invention, a method is provided for separating target particles from a mixture of target particles and non-

target particles, comprising (a) incubating the mixture of target particles and non-target particles in the presence of a first ligand capable of specifically binding to the target particles under conditions and for a time sufficient to allow binding of the first ligand to the target particles; (b) incubating the mixture of target particles and non-target particles in the presence of a first member chemically linked to a second ligand capable of binding to the first ligand under conditions and for a time sufficient to allow binding of the second ligand to the first ligand on the target particles; (c) passing the mixture of particles through a bed of low nonspecific binding porous material, the porous material having a second member capable of binding to the first member with an affinity constant of  $10^8 \text{ M}^{-1}$  immobilized on the surface thereof, wherein the pores of the porous material are of a size sufficient to allow the second member to enter into the pores and to allow entry of molecules greater than about 35 kD, but not so large as to allow collapse of the bed, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and (d) removing unbound non-target particles from the column.

Within a related aspect of the present invention, the bed of low nonspecific binding porous material has a second member capable of binding to the first member with an affinity constant of  $10^8 \text{ M}^{-1}$  immobilized on the surface thereof, the pores of the porous material being as large as possible with allowing collapse of the bed and of a size sufficient to allow the second member to enter into the pores. In addition, the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed.

Within another related aspect of the present invention, the mixture of particles is passed through a bed of low nonspecific binding porous polyacrylamide beads, the beads having a second member capable of binding to the first member with an affinity constant of greater than about  $10^8 \text{ M}^{-1}$  immobilized onto the surface thereof, wherein the pores of the beads are of a size sufficient to allow entry of molecules greater than about 60 kD into the pores, but not so large as to allow molecules of greater than about 300 kD into the pores. In addition, the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed.

Within yet another aspect of the present invention, a method for separating target particles from a mixture of target particles and non-target particles is provided. The method comprises (a) passing the mixture of particles through a bed of low nonspecific binding porous material, the porous material having a ligand capable of specifically binding to the target particles immobilized

on the surface thereof, wherein the pores of the porous material are of a size sufficient to allow the ligand to enter into the pores and to allow entry of molecules greater than about 35 kD, but not so large as to allow collapse of the bed, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and (b) removing unbound non-target particles from the column. Within a related aspect of the present invention, the bed of low nonspecific binding porous material has a ligand capable of specifically binding to the target particles immobilized on the surface thereof. The pores of the porous material are as large as possible without allowing collapse of the bed and of a size sufficient to allow the ligand to enter into the pores. The interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed. Within another related aspect of the present invention, the bed of porous material comprises a bed of low nonspecific binding porous polyacrylamide beads. The beads have a ligand capable of specifically binding to the target particles immobilized onto the surface thereof. The pores of the beads are of a size sufficient to allow entry of molecules greater than about 60 kD into the pores, but not so large as to allow molecules of greater than about 300 kD into the pores. The interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed.

These and other aspects of the invention will become evident upon reference to the following detailed description and attached drawings.

#### Brief Description of the Drawings

Figure 1 is an isometric view of a representative device of the present invention.

Figure 2 is a schematic illustrating a one-step method of the present invention.

Figure 3 is a schematic illustrating a two-step method of the present invention.

Figure 4 is a schematic diagram of a stem cell concentration system of the present invention.

#### Detailed Description of the Invention

As noted above, the present invention provides devices and methods for selectively isolating or separating target particles from a mixture of target particles and non-target particles. Particles within the mixture may be either positively or negatively selected. Positive selection refers to the direct selection

and recovery of specific target particles, while negative selection involves the elimination of a specific subpopulation of particles from a heterogeneous population of various particle types. Within the context of the present invention, target particles include, among others, viruses, bacteria, fungi, parasites and cells.

- 5 Cells include, among other types of cells, classes of human cells, such as endothelial cells, tumor cells, pancreatic islet cells, macrophages, monocytes, NK cells, B lymphocytes, T lymphocytes, and hematopoietic stem cells. Representative T lymphocytes include  $CD4^+$  cells,  $CD8^+$  cells, and specific subsets, such as  $IL2R^+$ ,  $CD19^+$ , and transferrin receptor  $TrR^+$  cells.
- 10 Hematopoietic stem cells include cells which may be selected on the basis of differentiation markers, for example, CD34.

The devices of the present invention generally comprise a column having a proximal end with an inlet port through which fluid may enter the column and a distal end with an outlet port through which fluid may exit the column, and a  
15 bed of low nonspecific binding porous material positioned within the column. The device may further comprise a means, located within the column, for agitating the porous material upon the application of an external force, such that target particles are released from the porous material.

A variety of column configurations may be suitable for use within  
20 the present invention. For example, a column may be configured in the traditional manner described above, or in various other forms, such as, for example, a radial flow column. A radial flow column is a packed bed confined within the annular space existing between two concentric porous cylinders. Fluid flow is arranged to occur radially across the bed from the outer cylinder to the lumen of the inner  
25 cylinder. A suitable radial flow column for use in the present invention is available from Sepragen Corp., San Leandro, California.

Columns for use in devices according to the present invention may be constructed using conventional molding techniques with materials including, among others, ABS, polypropylene, acrylics and polycarbonate (Acme Plastics,  
30 West Peterson, N.J.). If the column is to be used to obtain particles or cells for therapeutic uses, medical grade materials (USP class VI) are preferred.

Configuration of the column bed may be influenced by the particular target particle to be isolated. Column bed configuration is generally characterized by the access surface area and the bed depth. For purposes of the  
35 present invention, the access surface area is the geometrical surface area of the bed first presented to particles entering the column, and the bed depth is the distance traversed by the particles as they flow through the bed. The access

surface area is dependent upon two factors: total sample volume and total number of particles. As these factors increase, the access surface area should also be increased. The depth of the bed is dependent on particle size. As the particle size decreases, the bed depth, as well as the flow rate, should be increased in order to allow sufficient contact with the low nonspecific binding porous material. Column configuration is further dependent on whether highly purified particles (positive selection) or a high yield (depletion) of particles is preferred. If highly purified particles are preferred, the bed depth is decreased, such that only strongly bound particles will be immobilized. In contrast, if a high yield of target particles is desired, the bed depth is increased, thus allowing weakly bound target particles a better opportunity to become immobilized. Similarly, the capacity of the device is varied depending on the application. For positive selection (high purity) the total binding area of the affinity support in the device will be from 1 to 100 times the cross-sectional area of the particles multiplied by the total number of target particles. For depletion applications (high yield), the total binding area of the affinity support will be 50 to 10,000 times the cross-sectional area of the target particles multiplied by the number of such particles.

As discussed herein, the present invention provides devices and methods suitable for the separation of target particles from a mixture of target particles and non-target particles. The target particles may be separated in a direct method by passing them through a column containing a bed of low nonspecific binding porous material having a ligand capable of specifically binding to the target particles immobilized on the surface thereof. Alternatively, through a one-step or two-step method, the target particles may be bound directly or indirectly to a first member and passed over a bed of low nonspecific binding porous material which has a second member immobilized on the surface thereof. The second member is capable of binding to the first member with an affinity of greater than about  $10^8 \text{ M}^{-1}$ , and thus is capable of indirectly immobilizing the target particle onto the low nonspecific binding porous material. Various low nonspecific binding porous materials may function as a support, including, among others, porous hollow fibers (Amicon Corporation, Danvers, Mass.), beads (Polysciences, Warrington, Pa.), magnetic beads (Robbin Scientific, Mountain View, Calif.), meshes (Becton Dickinson, Mountain View, Calif.), screens and solid fibers (Edelman et al., U.S. Patent No. 3,843,324; and Kuroda et al., U.S. Patent No. 4,416,777). It will be evident to those skilled in the art that a variety of different sources exist for these porous materials other than those designated. As used herein, the phrase "low nonspecific binding" means that when the particles

are passed through  $5 \text{ cm}^2$  of porous material per million particles at a velocity of 1 cm/min in PBS plus 5% BSA buffer, less than 1% of the target and non-target particles (without ligand bound thereto) bind to the porous material in the column.

5 A preferred support is low nonspecific binding porous beads, wherein the pores of the beads are of a size sufficient to allow either the ligand in the direct method, or the second member in the one-step or two-step method, to enter into the pores. Within various embodiments the pore size is (a) of a size  
10 sufficient to allow entry of molecules greater than 35 kD, but not so large as to allow collapse of the bed; or (b) simply as large as possible without allowing collapse of the bed. Particularly preferred are polyacrylamide beads, such as Biogel™ (BIORAD; Richmond, Calif.). The pores of the polyacrylamide beads should be of a size sufficient to allow entry of molecules greater than 60 kD, but  
15 not so large as to allow molecules of greater than about 300 kD into the pores. As discussed herein, when polyacrylamide beads are utilized, the device includes means for retaining the beads within the column, for example, by a mesh or screen.

The low nonspecific binding porous material should also be chosen such that the interstitial spaces of the bed are of a size sufficient to allow the  
20 particles to pass through the column. This may be determined preliminarily by running a test sample of particles through the low nonspecific binding porous material in order to ensure that they can pass through the bed. In general, an interstitial space of about 2 to 20 particle diameters is preferred. This may be determined through microscopic analysis and measurement of the interstitial  
25 space and particle size. Where the porous material is beads and the particles are cells, bead sizes of  $170 \mu\text{m}$  to  $400 \mu\text{m}$  are generally preferred, with a  $250 \mu\text{m}$  bead size being particularly preferred. In general, the bead size decreases for smaller particles and increases for larger particles. Thus, a virus of about  $1 \mu\text{m}$  to  $2 \mu\text{m}$  may be passed through a column containing beads of about  $15 \mu\text{m}$  to  $50 \mu\text{m}$ ,  
30 whereas larger particles such as a parasite of about  $50 \mu\text{m}$  would require beads of about  $600 \mu\text{m}$  to  $2000 \mu\text{m}$ . These sizes however are preferable only for packed beds; bead size may differ for non-packed or fluidized beds. More specifically, non-packed or fluidized beds of beads require smaller bead sizes than a packed bed, in part due to the larger interstitial spaces which are attributable to the  
35 fluidity of the bed.

As noted above, within many aspects of the present invention, a second member is bound to the low nonspecific binding porous material. This

allows the indirect binding of the target cell to the low nonspecific binding porous material through either a one-step method involving a ligand-first member, or through a two-step method involving a ligand - anti-ligand - first member complex. In either case, the first member is capable of binding to the second member with an affinity of greater than about  $10^8 \text{ M}^{-1}$ . The second member may be selected from many first member-second member binding pairs, including, among others, biotin-avidin, biotin-streptavidin, biocytin-streptavidin, methotrexate-dihydrofolate reductase, 5-fluorouracil-thymidylate synthetase, riboflavin-riboflavin binding protein (see Becuar and Palmer, "The Binding of Flavin Derivatives to the Riboflavin Binding Protein of Egg White," J. Biol. Chem. 257(10):5607-17, 1982), antibody-protein A, and antibody-protein G. Either member of the above described binding pairs may function as the second member, with the complementary member functioning as the first member. Thus, either member may be attached to the low nonspecific binding porous material, with the complementary member being attached to the ligand if a one-step method is used, or to the anti-ligand if a two-step method is used.

In a preferred embodiment, a biotin-adsorbing group (the second member) is bound to the low nonspecific binding porous material, allowing separation of a biotinylated ligand through either the one-step or two-step methods. Suitable biotin-adsorbing groups include avidin, avidin analogs such as streptavidin, porin (see Korpela et al., "Binding of Avidin to Bacteria and to the Outer Membrane Porin of *Escherichia coli*," FEMS Microbiology Letters, 22, 1984) and combinations thereof.

A variety of methods may be used to immobilize the second member or biotin-adsorbing group onto the support. These methods include, among others, glutaraldehyde, carbodiimide, carbonyl diimidazole, cyanogen bromide, tosyl chloride, biotin/avidin, and biotin/streptavidin (see J.K. Inman, Methods in Enzymology, Vol. 34, Affinity Techniques, Enzyme Purification: Part B, W. B. Jakoby and M. Wilchek (eds.), Academic Press, New York, p. 30, 1974). Similarly, the "first-member" may be chemically linked to the ligand by any of a variety of methods well known in the art (see generally Wilchek and Bayer, "The Avidin Biotin Complex in Bioanalytical Applications," Analyt. Biochem. 171:1-32, 1988; see also Updyke and Nicolson, Methods in Enzymology, Vol. 121, "Immunoaffinity Isolation of Membrane Antigens with Biotinylated Monoclonal Antibodies and Streptavidin-Agarose," J.J. Langone and H. VanVunakis (eds.), Academic Press, New York, pp. 717-25, 1986).



Within another aspect of the present invention, the ligand may be directly coupled onto the support by the methods discussed above. Such a device allows the direct separation of target particles by incubation with the immobilized ligand, as discussed below.

5 A particularly preferred embodiment of a device according to the present invention may be more precisely described through reference to Figure 1. As shown in Figure 1, the device 10 is comprised of a cylindrical column 12 having a proximal endcap 14 with an inlet port 18 and a distal endcap 16 with an outlet port 20. The endcaps 14 and 16 have a means 26, such as a support surface, which  
10 support a mesh 24. The mesh is used to retain a bed of porous beads within the column. The endcaps also have ribs 22 which provide uniform flow distribution with a minimum flow volume. The column, endcaps and ribs are preferably made of a thermoplastic material, for example, injection molded polycarbonate. Positioned between the mesh supports is a bed of low nonspecific binding porous  
15 polyacrylamide beads 28 and a magnetic starburst impeller 30 for agitation of the beads. In the proximal endcap, the ribs 22 and mesh 24 direct the flow of fluid entering through the inlet port 18 from the center of the device and distribute the flow over the entire surface of the bed 28 rather than allowing the flow to concentrate in the middle or center of the bed. The ribs 22 in the distal endcap  
20 channel the fluid flow toward the outlet port 20. Preferably, the column is 4 cm in diameter with a space between the mesh of 3.8 cm in which the beads and impeller are retained. The polyacrylamide beads preferably have a particle size of on average, 250  $\mu$  and a pore size of greater than 60 kD but less than 300 kD. Avidin is bound to the surface of the beads at a concentration of between 25 and  
25 1,000  $\mu$ gr/ml of gel, preferably a concentration of 500  $\mu$ gr/ml of gel.

As noted above, the device may also include a means for agitating the low nonspecific binding porous material upon the application of an external force, such that bound target particles are released. The means for agitation may be used to remove bound target particles from affinity materials which are low  
30 nonspecific binding and either nonporous or porous. Means for agitating the affinity material may be located directly in the column, and include magnetic impellers (Parker Industries Corporation, Chicago, Ill.), magnetic beads (Robbin Scientific, Mountain View, Calif.), weights (Astrolite Alloys, Camarillo, Calif.), magnetic weights, pipettes, and buoyant floats (Polysciences Corp., Warrington,  
35 Pa.). The external force which supplies energy to the means for agitating the affinity material is generally located outside of the column and includes electromagnetic, gravitational and mechanical forces. Electromagnetic forces may

act on agitating means such as metallic or magnetic beads or weights, causing agitation and release of particles. Electromagnetic forces may be provided by, for example, magnetic stir plates (VWR Scientific, San Francisco, Calif.) or electromagnetic coils (Radio Shack). Gravitational forces may be provided by, for example, rocking plates or manual shaking of a column, such that the forces of gravitation agitate the affinity material, thereby causing release of particles. A weight which is either denser or more buoyant than the affinity material may be placed in the bed to enhance the effect of gravitational forces on bed agitation. Mechanical forces may be provided by, for example, hydrodynamic jets (VWR Scientific, San Francisco, Calif.), vortexes (VWR Scientific, San Francisco, Calif.), or sonicators (Curtin Matheson Scientific, Seattle, Wash.). To determine the proper level of agitation using these means, the level of force is gradually increased until the affinity material is visibly moved. This level of agitation may then be fine-tuned; for example, a lower force may be utilized to remove only nonspecifically bound particles followed by a greater force to remove the most tightly bound particles.

In addition to the above-described device, the present invention provides a system for the processing of target and non-target particles. The system may include a prefilter to remove particulates, aggregates, or other debris prior to passing the sample over the bed of low nonspecific binding porous material. Various materials may be used as a prefilter, including, for example, blood filters (Cutter Biologicals, Berkeley, Calif.). Particularly preferred is low nonspecific binding porous material, as discussed above (without an immobilized second member or ligand thereon), which is used as a prefilter prior to the use of the immobilization column.

Other system components may include valves or clamps which function to control the flow of fluid. Within a preferred embodiment, the flow of fluid is controlled by a microprocessor, and monitored by various optical sensors (Optek Technology, Inc., Carton, Tex.). Other system components may include peristaltic pumps (Curtin Matheson Scientific, Seattle, Wash.) and pressure transducers (Sensym, Sunnyvale, Calif.).

Within a related embodiment of the present invention, the column is part of a closed system which allows the separation or isolation of target particles from a mixture of particles in a sterile manner, *i.e.*, such that external organisms do not enter the mixture. Such a closed system comprises a sterile column having a proximal end with an inlet port through which fluid enters the column and a distal end with an outlet port from which fluid exits the column; a

sterile bed of low nonspecific binding porous material located within the column; a sterile magnetic impeller located within the column; a means for introducing a mixture of cells, as well as sterile process fluids into the column, while maintaining sterility; a means for collecting cells and process fluids exiting from the column, while maintaining sterility; and an external source of electromagnetic force for regulating the rotation of the impeller.

The closed system may be sterilized by any method known in the art, including electron beam (EB) radiation,  $\gamma$  irradiation, or ethylene oxide. Each of these methods may result in some loss of activity of the immobilized species. Furthermore, the low nonspecific binding material may be preserved through the use of an agent such as sodium azide, benzoic acid, sorbate, or dehydroacetic acid. In a preferred embodiment, avidin-conjugated polyacrylamide beads are preserved with 1% benzoic acid, followed by sterilization with 2.5 megarads of electron beam radiation.

A preferred sterile closed system is illustrated with reference to Figure 4. As shown in Figure 4, a stem cell concentration system is depicted having a series of bags 46, 47 and 48 for introducing process fluids into the system in a sterile manner. The stem cell mixture is contained in a mixing bag 46. The mixing bag 46 is connected to a first, three-way pinch valve 53 with polyvinylchloride (PVC) tubing 51. The PBS plus BSA buffer bag 47 is connected to a spike port 50 which is connected to PVC tubing 51 leading to a second, three-way pinch valve 54. The PBS bag 48 is connected to a spike port 50 which is connected to the second three-way pinch valve 54 with PVC tubing 57. The PVC tubing 51 leading from the bags 46, 47, and 48 are closed off with clamps 52 during set up. The flow of process fluids from three-way pinch valves 53 and 54 is controlled by optical sensor 56 coupled with a microprocessor (not shown). The optical sensor indicates when process fluids have passed to the microprocessor, which switches the valves to the appropriate on or off positions. PVC tubing 51 leading from the optical sensor 56 is threaded through a peristaltic pump 58 which regulates the rate of flow of process fluids. Connected to the tubing 51 threaded through the pump 58 is a  $0.22\ \mu$  filter 60 for venting air from the system. The tubing 51 leading from the pump 58 is connected to a spike port 50 which is connected to a third three-way pinch valve 61. A prefilter 64 is connected to a three-way pinch valve 61 with PVC tubing 51. The prefilter 64 is connected to a separation device 10 with tubing 51. Also provided is a bypass loop 62 of PVC tubing which is connected to the third three-way pinch valve 61 and to the tubing 51 leading from the prefilter 64. The separation device 10 has a bed of porous

material 28 for adsorbing the target stem cells. Within the device 10 is a magnetic impeller 30 for agitating the porous material. The device 10 is supported on a magnetic stir plate 65 which regulates the rotation rate of the magnetic impeller 30. Tubing 51 leading from the device 10 is threaded through the stir plate 65 and is connected to a spike port 50 which is connected to tubing 51 leading from the spike port to an optical sensor 55 coupled with a microprocessor (not shown). Tubing 51 from the optical sensor 55 is connected to a fourth, three-way pinch valve 57. Connected to three-way pinch valve 57 is a waste bag 66 for receiving process fluids and a collection bag 68 for recovering stem cells released from the porous material by agitation. The optical sensor 55 indicates when fluids have passed to a microprocessor which switches three-way pinch valve 57, directing fluids to either the waste bag 66 or collection bag 68. The tubing 51 connecting pinch valve 57 to the collection bag 68 is shut off with a clamp 52 during set up. After set up is completed, the clamps 52 can be removed.

Also provided by the present invention are methods for separating target particles from a mixture of target and non-target particles, utilizing the devices discussed above. In general, the methods comprise the steps of (1) incubating the target and non-target particles with either an immobilized ligand, or a ligand which will subsequently be immobilized, such that the ligand binds to and hence functions to immobilize the target particles, and (2) removing unbound non-target particles from the column.

As discussed above, various particles may be separated using the methods of the present invention, including, among others, viruses, bacteria, fungi, parasites, and cells. Some of the above particles may be found within biological samples or specimens. Thus, it may be advantageous to preliminarily prepare such samples (see D.A. Lennette, "Collection and Preparation of Specimens for Virological Examination," Manual of Clinical Microbiology, pp. 687-693, 1985; and McIntosh et al., "Influence of Tumor Carriage on the Production of Lymphokines Affecting Macrophage Behavior," Immunology 57:359-365, 1986). For example, stool samples contain a plethora of bacteria, as well as other particles such as parasites, and may be prepared suitable for use within the present invention utilizing conventional techniques (see J.W. Smith and S.M. Bartlett, "Diagnostic Parasitology: Introduction and Methods," Manual of Clinical Microbiology, pp. 595-611, 1985). Briefly, the stool sample is strained through gauze into a 15 ml tube. Saline is added and the suspension is centrifuged at 650 g for 3 minutes. The supernatant is poured off, and the sample is incubated with an

anti-parasitic antibody. The sample is then passed over a column as described below in order to immobilize antibody bound parasites.

Particles may also be preliminarily treated by passing them through a prefilter, as described above, prior to passing them over a column for  
5 immobilization. The prefilter functions to remove particulates and aggregates that would otherwise block the interstitial spaces of the immunoadsorption column.

As noted above, various methods may be used to immobilize the target particles, including use of an immobilized ligand, a one-step method, or a  
10 two-step method. If an immobilized ligand is used, the particles may be passed directly over the low nonspecific binding porous material under conditions and for a time sufficient to allow binding to occur. More specifically, the passage rate of particles through the column should be sufficiently slow such that the particles are in contact with the surfaces containing the immobilized ligand for a time sufficient  
15 to allow specific binding to occur, preferably at least 15 minutes. A ligand is defined to be "specifically binding" when it binds to more than about 90% of the target particles, but to less than about 1% of the non-target particles. This may be readily determined through the use of a Fluorescence Activated Cell Sorter (FACS).

20 Various ligands may be utilized within the present invention, the choice of ligand being dependent upon the target particle of interest. The binding of the ligand to the particle is usually dependent on certain moieties or substrates which are present on the particle. General ligand-particle substrate pairs include: lectin-carbohydrate (for example, wheat germ agglutinin - N-acetyl glucosamine;  
25 and concanavalin A - glucose or mannose); enzyme inhibitor-enzyme (for example, organophosphate-acetyl cholinesterase); binding protein-receptor (for example, fibronectin-gp1c, or collagen-gp1a); substrate molecule-transport protein (for example, cyclophosphamide - multi-drug resistance protein); hormone-receptor (for example, transferrin-transferrin receptor); and vitamin-binding  
30 protein (for example, biotin-avidin). Particularly preferred ligand-particle substrate pairs include antibody-antigen pairs. Monoclonal antibodies may be produced using conventional techniques (see generally H. Zola (ed.), Monoclonal Antibodies: A Manual of Techniques, CRC Press, Boca Raton, Fla., 1987) or obtained from conventional sources. For example, a monoclonal antibody specific  
35 for Ia antigens suitable for use within the present invention may be made according to the method of Hansen et al. (Immunogenetics 10:247-60, 1980), or purchased from a conventional source such as the American Type Culture

Collection, Rockville, Maryland (see ATTC Nos. HB55, HB96, and HB104) and utilized to bind Ia bearing cells. Similarly, a monoclonal antibody may be produced to a parasitic antigen as described by Perlmann et al. ("Antibodies in Malaria Sera to Parasite Antigens in the Membranes of Erythrocytes Infected with  
5 Early Asexual Stages of *Plasmodium falciparum*" J. Exp. Med. 159:1686-1704, 1984), and used within the present invention to detect the presence of parasites. Monoclonal antibodies may also be obtained from commercial sources. For example, a monoclonal antibody such as Anti-HPCA-1 (Becton Dickinson, Mountain View, Calif.) may be used within the present invention to bind stem  
10 cells which have CD34 antigens.

In addition, an entire antibody need not be used as the ligand; only the binding region of the antibody is necessary to specifically bind to particles. Thus, antibody fragments such as Fab or F(ab')<sub>2</sub> fragments may be used within the present invention. Furthermore, the binding regions of the specifically binding  
15 antibody may be incorporated into a new protein, which may be used as the ligand (see Reichmann et al., "Reshaping Human Antibodies For Therapy," Nature 332:323-327, 1988; Verhoeyen et al., "Reshaping Human Antibodies: Grafting an Antilysozyme Activity," Science 239:1534-1536, 1989; and Roberts et al., "Generation of an Antibody With Enhanced Affinity and Specificity for its  
20 Antigen by Protein Engineering," Nature 328:731-734, 1987).

Within another aspect of the present invention, a one-step method may be used to immobilize and separate target particles from a mixture of target particles and non-target particles. Within this aspect, a mixture of target particles and non-target particles is incubated in the presence of a first member which is  
25 chemically linked to a ligand. The ligand is capable of specifically binding to the target particles under conditions as discussed above. In a preferred embodiment, the mixture of target and non-target particles are incubated with a 100-fold excess of ligand, such that essentially every binding site of the particle is occupied. The mixture is then passed over a column containing the low nonspecific binding  
30 porous material which is coupled to a second member. The second member is capable of binding to the first member with an affinity of greater than about  $10^8 \text{ M}^{-1}$ , thus allowing immobilization of the target particles. Unbound or non-binding particles may then be removed, and the bound particles subsequently released by agitation as discussed above.

35 Various embodiments of the one-step method have been discussed above, including representative ligands and first member-second member binding pairs. In addition, multiple combinations of the first member-second member

binding pair may be employed. For example, biotin may be linked to the ligand, as well as adsorbed to the low nonspecific binding porous material surface. The particle-ligand-biotin complex and biotin-support complex may then be bound together through incubation with avidin. Avidin is multivalent, permitting the  
5 formation of a particle-ligand-biotin-avidin-biotin-support complex which immobilizes the particle.

A representative one-step method of the present invention can best be illustrated with reference to Figure 2. A mixture of target cells 32 and non-target cells 34 are incubated with biotinylated antibody 36, yielding a mixture of  
10 target cells bound to the biotinylated antibody 32a and non-target cells 34a. The mixture of cells is passed through a separation column 10 containing a bed of porous beads 38. The porous beads have avidin 40 immobilized on the surface thereof. As the mixture of cells is passed through the column 10, the cells come into contact with the porous beads 38. As a consequence, the target cells bound to  
15 biotinylated antibody are adsorbed to avidin on the porous beads. The adsorbed cells 32b are retained in the column 10 and non-target cells 34b pass through the column, exiting through the outlet port 20, and can be recovered as unadsorbed, non-target cells 34c, along with free excess biotinylated antibody 36. Some of the excess biotinylated antibody 36a is trapped in the column and is adsorbed onto the  
20 porous beads. After the unadsorbed, non-target cells 34c have passed through the column, the bound or adsorbed target cells 32b can be recovered by agitation, providing released target cells 32c.

Within a preferred embodiment, the target particle is a stem cell which can be found circulating in peripheral blood. Briefly, a sample of the blood  
25 is incubated with an excess amount of a ligand which is chemically linked to a first member, such as a biotinylated anti-CD34 antibody. Anti-CD34 antibodies may be obtained from various suppliers including Becton Dickinson, Mountain View, Calif., and Quantum Biosystems, Waterbeach Cambridge, U.K. Most monoclonal antibodies can be biotinylated according to the method of Updike and Nicolson  
30 ("Immunoaffinity Isolation of Membrane Antigens with Biotinylated Monoclonal Antibodies and Streptavidin-Agarose," Methods in Enzymology 121:717-725, 1986). The biotinylated anti-CD34 antibody specifically binds to the stem cells. The sample is then passed over a packed bed of porous material having a pore size as discussed above, upon which the second member is immobilized. In this  
35 case, the second member is immobilized avidin. The cell, biotinylated antibody complex is adsorbed to the immobilized avidin, thus allowing the subsequent removal of unbound blood products as discussed above.

Within yet another preferred embodiment, the target particle is a nucleated erythroid fetal cell which can be found circulating in maternal blood (see related application entitled "A Method for Enriching Fetal Cells From Maternal Blood," attorney's docket number 200072.402, which is incorporated  
5 herein by reference). Briefly, fetal nucleated erythroid cells may be enriched by incubating a sample of maternal blood with a first member such as biotin which is chemically linked to a ligand, such as an antibody, which is capable of specifically binding to fetal nucleated erythroid cells. The cells are then adsorbed to an immobilized second member such as avidin, followed by the removal of unbound  
10 blood products. Further enrichment may be performed either through a method utilizing erythropoietin, and/or through a method utilizing ammonia, chloride ions, and a carbonic anhydrase inhibitor. This method results in the enrichment of fetal cells such that hybridization assays or chromosomal analysis may be performed.

15 Within a related aspect of the present invention, a two-step method is used to separate target particles from non-target particles. Briefly, a first ligand is incubated with a mixture of target and non-target particles. As discussed above, the ligand is chosen so as to specifically bind to target particles (under suitable conditions, as described above). Subsequently, a second ligand which has been  
20 chemically linked to a first member is added. The second ligand is capable of binding to the first ligand. The particle, first ligand, second ligand, first member complex may then be adsorbed onto an immobilized second member, thus allowing the separation of target particles from unbound non-target particles. Representative examples of the first ligand, and of the first member-second  
25 member binding pair have been discussed above. The second ligand should be chosen such that it is capable of specifically binding to the first ligand; examples include antibodies, protein A, and protein G. A particularly preferred second ligand is an antibody which specifically binds to the first ligand. For example, if the first ligand is an antibody, a suitable second ligand would be an anti-  
30 immunoglobulin antibody, which is available from a number of conventional sources (*e.g.*, Sigma Chemical Company, St. Louis, Mo.).

Within a preferred embodiment of the present invention, the mixture of particles is incubated with an excess amount of a first member chemically linked to a second ligand. Any unbound second ligand-first member  
35 may then be removed by techniques well known in the art, for example, by centrifugation and resuspension.



A two-step method of the present invention can be illustrated with reference to Figure 3. A mixture of target cells 32 and non-target cells 34 are incubated with an antibody 42. The antibody binds to the surface of the target cell, producing antibody bound target cells 32d and non-target cells 34d. The mixture of cells is next incubated with biotinylated anti-antibody 44, to provide a mixture containing non-target cells 34e and target cells 32e bound to antibodies 42 that, in turn, are bound to biotinylated anti-antibodies. This mixture of cells is then passed through a column 10 containing avidin 40 coated beads 38. The target cells 32e are adsorbed to the porous beads, providing adsorbed target cells 32f. The non-target cells pass through the column as non-target cells 34f and out the outlet port 20, yielding a mixture containing unadsorbed non-target cells 34g, free biotinylated anti-antibody/antibody and free biotinylated anti-antibody. The adsorbed target cells 32f are released by agitation providing released target cells 32g. Excess biotinylated anti-antibody/antibody 43 and excess biotinylated anti-antibody 45 may be trapped by the avidin 40 on the porous beads 38.

As noted above, once the target particles have been immobilized, unbound non-target particles may be removed. In one embodiment, once the target particles are bound to the support, the column is rinsed with a buffer, removing the unbound non-target particles. A variety of suitable buffers are well known in the art, including PBS, PBS plus albumin (such as Bovine Serum Albumin (BSA)), normal saline, and cell culture media.

The methods of the present invention may be performed using additional steps such as washing unbound ligand or ligand-first member out of the system, prior to exposure to the immobilized second member. Various methods may be used to perform this wash step, although centrifugation of the particles followed by resuspension in buffer is generally preferred.

Within preferred embodiments of the present invention, the target particles are released from the porous material. Various methods for releasing particles may be utilized, including, among others, agitation of the low nonspecific binding porous material; culturing particles such as cells with or without cytokines; and cleavage of the ligand or particle-ligand bond. More specifically, agitation may be performed using electromagnetic, gravitational, or mechanical forces acting upon the porous material or components within the bed such as a magnetic impeller. Particles such as cells may also be removed from the porous materials by simply culturing the cells. Many cells change during culture such that they or their progeny are released from the surface. This process can occasionally be accelerated by adding appropriate growth factors to the culture media. For

example, addition of IL-2 accelerates the release of T cells bound to surfaces. Particles may also be released from the porous material by chemical cleavage of the ligand or the ligand-particle bond. One example is the use of papain to digest antibodies binding particles to the surface.

5           Within a preferred embodiment, particles are fractionally released from the porous material. Fractional release allows separation and collection of particles based upon the strength of their bond to the support. Thus, particles with lower overall bond strength will be released with lower amounts of an applied external force, whereas particles which are more strongly bound will not be  
10 released without greater amounts of an applied external force. Thus, incrementally increasing the external force allows fractional release of particles based upon the relative strength of their bonds to the support. The external force required to cause release will be dependent upon, among other aspects, the size of the particle. In general, smaller particles, for example, viruses, require less actual  
15 energy to be released because of their smaller size, and hence fewer numbers of bonds to the support surface. However, because of the small size of the virus, a large shear force must be applied to the system. Thus, the external force required to release a virus may require about 10 to 100 times the energy required to release a cell. In contrast, particles which are larger than cells, for example, parasites,  
20 require more actual energy to be released due to the large number of bonds to the support. However, because of the larger size, shear forces are more readily absorbed by parasites, resulting in approximately 20 times less external force being required to release the parasite as compared to a smaller particle such as a cell.

          A variety of methods exist for agitating the bed to cause fractional  
25 release. For example, a weight may be placed in the column, and the column inverted several times. The movement of the weight through the column agitates the bed, thus causing release of bound particles. Similarly, magnetic beads may be used as the bed itself. Once particles have been immobilized on the bed and washed, they may be fractionally released by movement of the beads with an  
30 electromagnetic force, such as an electromagnetic coil.

          Within a preferred embodiment, cells are fractionally released by an impeller in a 4-cm diameter column. Briefly, the impeller is run at 1 rpm for a period of time long enough to collect 1 to 3 column volumes of PBS buffer (50 to 150 ml). This wash solution will contain most of the nonspecifically bound cells.  
35 Subsequently, the impeller is accelerated (e.g., to 25 rpm) to collect specifically bound cells which are larger or which have less ligand binding sites. After

collecting 1 to 3 column volumes, the impeller speed is again increased to collect cells which are more tightly bound.

FACS analysis of cells which have been separated utilizing a biotinylated antibody, and a column containing avidinated polyacrylamide beads following the procedures described below, result in the removal of at least 25% and usually 70% to 90% of the biotinylated antibody from the surface of the cells, and yet allows greater than 90% of the cell surface antigens or receptors to remain. General procedures are described in a related application entitled "Methods For Removing Ligands From a Particle Surface," attorneys' docket number 20072.403, which is incorporated herein by reference.

The following examples are offered by way of illustration and not by way of limitation.

## EXAMPLES

### EXAMPLE 1

#### ISOLATION OF STEM CELLS

##### A. PREPARING THE BUFFY COAT CELLS

A sample of bone marrow is centrifuged at 240g for 15 minutes. The plasma is removed, and the remaining buffy coat cells are centrifuged once more at 240 g for 15 minutes in order to remove red blood cells. The buffy coat cells are washed twice with PBS by centrifugation at 280g for 10 minutes. The cells are then resuspended to a final concentration of  $1 \times 10^8$  white cells/ml in PBS plus 1% BSA.

##### B. INCUBATION OF BUFFY COAT CELLS WITH ANTIBODY

The suspension of buffy coat cells is incubated with a final concentration of 10  $\mu$ g/ml biotinylated anti-CD34 antibody (Quantum Biosystems, Waterbeach Cambridge, U.K.) at 4°C for 10 minutes. The antibody-cell mixture is then washed twice with PBS plus 1% BSA by centrifugation at 280 g for 10 minutes. The cells are then resuspended at a concentration of  $1 \times 10^8$  white cells/ml in PBS plus 5% BSA.

### C. CARBOXYLATION OF A POLYACRYLAMIDE GEL

Seventeen grams of dry Biogel P-60™, (50-100 mesh (wet), coarse beads) (BIORAD, Catalog No. 150, 1630, Richmond, Calif.) are added to 1.5 l of 0.5 M NaHCO<sub>3</sub>/0.5 M Na<sub>2</sub>CO<sub>3</sub>. The pH is adjusted to 10.5 with NaOH and carefully stirred with a mixer (RZR1, Carfamo, Wiarnton, Ontario, Canada) so as not to damage the beads for approximately 20 to 30 minutes. The mixture is then placed in a 60°C water bath. After the mixture reached a temperature of 60°C, it is incubated for an additional 2 hours (at 60°C) with occasional stirring. The mixture is then removed from the water bath, and placed in an ice bath to bring the mixture temperature down to room temperature.

The beads are washed several times with distilled or deionized water, followed by several washings with PBS using a coarse glass filter connected to a vacuum. The carboxylated gel may be stored in PBS at 4°C, and is stable for up to one year if sterilized or stored with a preservative.

### D. AVIDIN CONJUGATING THE CARBOXYLATED BIOGEL

PBS is first removed from a measured amount of carboxylated Biogel by filtering with a coarse glass filter connected to a vacuum. The gel is then equilibrated in distilled or deionized water for 15 to 30 minutes. Equilibration in water causes an expansion of the gel to a volume of about 4 times its previously measured amount. The gel is resuspended in 10 ml of distilled or deionized water for each ml of gel (as originally measured in PBS).

Twenty mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC-HCl) (Sigma Chemical Co., Catalog No. E7750, St. Louis, Mo.) is added for each ml of gel as originally measured. The pH is rapidly adjusted to 5.5 by dropwise addition of HCl. Care is taken to maintain the pH at 5.5; pHs of less than 5.0 or greater than 6.0 result in significantly less activation of the Biogel. The mixture is stirred for five minutes.

Avidin (International Enzymes, Inc., Fallbrook, Calif.) is dissolved at a concentration of between 10 and 100 mg/ml in deionized water. Next, 1 mg of avidin is rapidly added for each ml of gel (as originally measured in PBS). The mixture is stirred for 1.5 hours. Next 2 M glycine is added to give a final concentration of 0.2 M glycine in the mixture, and stirred for an additional 1 hour.

The gel is washed with several volumes of PBS using a coarse glass filter and vacuum, and stored in PBS with 0.1% NaN<sub>3</sub> at 4°C. The gel is stable for approximately one year.

#### E. COLUMN PREPARATION

The column is attached to a prefilter. The column is supported on a stir plate. A cell collection bag and waste bag unit are attached to the column by threading the tubing of the unit through the stir plate, and spiking into the outlet port of the column. Inlet tubing is attached to the inlet port of the prefilter. The other end of the inlet tubing is inserted into a pump. The PBS bag and the PBS plus 5% BSA bag are attached to the inlet tubing exiting the pump using spikes. In order to begin operation air is purged from the tubing by opening the valve to each bag and pumping media through the tubing. Air is vented out of the 22 micron filter which is situated between the pump and the prefilter. The valves from the PBS plus 5% bag are opened and the contents thereof are pumped through the column at a rate of 10 ml/min. into the waste bag, flushing the system and removing air from the tubing and affinity gel. An impeller in the column is activated at 30 rpm for the first five minutes of the flushing step. After the impeller is turned off, the pump is allowed to run for another 10 minutes.

#### F. COLUMN OPERATION

The incubated cell suspension is pumped through the column as described above at a rate of 10 ml/min. The valve to the mixing bag is closed when the last cells are exiting the mix bag so air contained therein does not enter the system. The valve to the PBS plus 5% BSA bag is opened and the media is pumped through the column at a rate of 10 ml/min. After 5 minutes, the valve to a bypass loop around the prefilter is opened. The valves are then switched to allow the media of the PBS bag to flow through the column. The impeller is turned on at 1 rpm after the PBS wash runs for five minutes. After five minutes, the valves are switched so that the flow from the column is into the collection bag and the impeller rate is increased to 80 rpm. After 10 minutes the impeller and pump are turned off. The tubing to the collection bag is sealed and cut free from the system for transport or further handling.

#### G. RESULTS

Of the approximately 25 billion bone marrow cells that are passed through the column, 280 million of the cells are bound to the column and are recovered in the collection bag. Viability of the collected cells is 91% as measured by trypan blue exclusion.

A sample of the collected cells is incubated for 30 minutes at 4°C with 20 µg/ml fluorescein labeled goat-anti-mouse (IgG + IgM) antisera F(ab)'<sub>2</sub>

fragment (Tago, Inc., Burlingame, Calif.). After washing by centrifugation at 280 g for 8 minutes, the cells are analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.). The FACS analysis shows that 58% of the collected cells are CD34 positive.

5

## EXAMPLE 2

### EFFECT OF PORE SIZE IN THE POROUS MATERIAL

10

#### A. INCUBATION OF BUFFY COAT CELLS WITH ANTIBODY

15 Buffy coat cells are prepared from bone marrow as described above in Example 1. A final concentration of 10  $\mu\text{g/ml}$  of anti-CD34 antibody is added to the buffy coat cell suspension and incubated at 4°C for 30 minutes. The antibody-cell mixture is washed twice with PBS + 1% BSA by centrifugation at 280 g for 10 minutes at 4°C. The cells are resuspended in PBS + 1% BSA to a concentration of  $10^8$  cells/ml and incubated with a final concentration of 1  $\mu\text{g/ml}$  of biotinylated goat-anti-mouse antisera (Southern Biotech, Birmingham, Alabama) for 30 minutes at 4°C. The cells are washed twice with PBS + 1% BSA by centrifugation and resuspended at 50 million cells/ml in PBS + 5% BSA.

20

#### B. PREPARATION OF AVIDIN COATED POROUS BIOGEL

25 Biogel P-30 and Biogel P-60 are prepared as described above in Example 1, except that 10 mg of EDC-HCl and 100  $\mu\text{g}$  of avidin is added per ml of gel instead of 20 mg EDC-HCl and 1 mg avidin. Biogel P-30 is carboxylated as in Example 1, part C, for use in precolumns.

#### C. COLUMN PREPARATION

30 K9/15 columns (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) are assembled with 80 micron nylon mesh in the endcap. Tubing is fitted to the endcap and threaded through a peristaltic pump (Cole Parmer, Chicago, Ill.) to a collection tube. Avidin coated Biogel prepared above is added to the column in PBS and settled to a depth of 4 cm. Air bubbles in the gel bed are removed by agitation with a pipette. The gel in the column is washed and equilibrated with PBS + 5% BSA. Precolumns are made from K9/15 columns as above except that 35 they are filled with a 1 cm bed of carboxylated P-30 gel and the tubing from the bottom of each precolumn leads into the top of the avidin Biogel-filled column

immediately below. The liquid level in each column is drawn down to the level of the gel.

#### D. COLUMN OPERATION

5           Three hundred million bone marrow cells treated above are gently layered onto the top of the carboxylated gel in the precolumn and allowed to flow by gravity into the avidin-coated Biogel column below at about 1 ml/min. The cell mixture is pumped through the avidin-coated Biogel column at 1 ml/min. The precolumn is washed with 1-2 mls of PBS + 5% BSA after the cell solution passes  
10 through. As the last of the cell solution enters the avidin-coated gel, two ml of PBS + 5% BSA is gently added to that column to wash residual cells through the bed. Before the last of the PBS + 5% BSA level enters the bed, PBS is layered gently on top of the solution, forming an interface. The bed is washed with an additional 8 ml of PBS before adherent cells are recovered. The adherent cells  
15 are released from the gel by agitating the gel through a pipette while flowing PBS at about 3 ml/min. through the column.

#### E. RESULTS

20           The cells are analyzed as described in Example 1. As shown below in Table 1, the larger pore size gel unexpectedly gave significantly better yield of CD34 positive cells and much greater purity.

Table 1

25				
	Pore Size	CD34 + Cells (million)	% Purity	% Viability
	P-30	1.2	61	98
30	P-60	2.5	77	93

#### EXAMPLE 3

35

#### EFFECT OF PORE SIZE ON PERFORMANCE

A. PREPARATION OF BUFFY COAT CELLS AND INCUBATION  
WITH BIOTINYLATED ANTI-IA ANTIBODY

Buffy coat cells are prepared from peripheral blood as described in

Example 1.

5 Anti-Ia antibody is biotinylated using 2mM sulfo-NHS-biotin (0.887 mg/ml in pH 8.0 0.1 M HEPES) (prepared immediately before use), and 0.1 M HEPES pH 8.0. Briefly, 1 mg of purified monoclonal antibody (at a concentration of 2-5 mg/ml) is placed in a polypropylene tube and brought to a final volume of 0.932 ml with 0.1 M HEPES. Sixty-eight  $\mu$ l of sulfo-NHS-biotin  
10 stock is added, vortexed, and incubated at 0°C for 4 hours. The reaction mixture is transferred to a Centricon 30 microconcentrator (Amicon, Danvers, Mass.) and washed five times with 2 ml aliquots of 0.1 M HEPES plus 0.05% NaN<sub>3</sub>. The mixture is reconstituted to 2-5 mg/ml in 0.2 M HEPES, 50% (v/v) glycerol, and 0.05% NaN<sub>3</sub> and stored at -80°C.

15 Biotinylated anti-Ia antibody is incubated with the buffy coat cell suspension at 4°C for 10 minutes. The antibody-cell mixture is then washed twice with PBS plus 1% BSA by centrifugation at 280 g for 10 minutes. The cells are then resuspended at a concentration of  $1 \times 10^8$  white cells/ml in PBS plus 5% BSA.

20

B. PREPARATION AND OPERATION OF THE COLUMN

A variety of different gels (including Biogel P-6, P-30, P-60, P-100, P-200, and P-300) are carboxylated as described in Example 1, and avidinated as also described above with 20 mg/ml EDC and 1 mg/ml avidin. Columns  
25 containing the various gels are prepared and operated as described in Example 2.

C. RESULTS

The results are analyzed in the same manner as described in Example 1, except that the cells are stained with additional anti-Ia antibody (30  
30 min. at 4°C), washed, and then stained with 20  $\mu$ g/ml fluoresceinated goat-anti-mouse antibody for FACS analysis. The results are displayed in Table 2 below.



Table 2

	Pore Sieve Size (DALTONS)	Yield	Purity
5	6,000	6.2%	90%
	30,000	13.4%	95%
	60,000	28.7%	92%
	100,000	31.6%	96%
	200,000	23.5%	89%
10	300,000	30.0%	90%

In contrast to conventional techniques which dictate as small a pore size as possible, the present invention demonstrates improved yield and purity with very large pore sizes.

#### EXAMPLE 4

##### REMOVAL OF BOUND CELLS WITH AGITATION

###### A. CELL PREPARATION AND COLUMN OPERATION

Buffy coat cells are prepared and incubated with a biotinylated anti-Ia antibody at 1  $\mu\text{g/ml}$  as described in Example 3.

Biogel P-60 is carboxylated and conjugated with avidin as described in Example 2, and placed in a Pharmacia K9/15 column except that a stainless steel sheet metal screw weighing 1.1 gm is placed in the gel bed before the top screen, mesh, and end caps are applied. The column is operated as described in Example 3, except that after the cells had been washed on the column with PBS, the column is inverted and righted one time so that the stainless steel screw traveled from the bottom to the top of the column and back again thus agitating the column bed. The PBS which flowed through the column during the agitation is collected in a separate tube and analyzed as described in Example 1. The analysis of the collected cells is given below as "once." After collecting the "once" fraction, the column is inverted and righted steadily for 15 minutes. The cells collected during this interval are analyzed and the results are given below as "steady."

**B. RESULTS**

The size of the cells is analyzed by using the FACScan instrument to measure the amount of light scatter. The cells are classified as either small (the size of lymphocytes) or large (the size of monocytes). The results are indicated in

5 Table 3 below.

Table 3

10	Fraction	Total % Purity	% Small	% Large
	Once	90.0	32.6	57.4
	Steady	93.0	70.8	22.2

15 As indicated by the data, larger cells are removed with very little agitation, whereas smaller cells take more extensive agitation in order to be removed.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for the purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by  
20 the appended claims.

Claims

1. A device for the separation of target particles indirectly bound to a first member from a mixture of target particles and non-target particles, comprising:

a column having a proximal end with an inlet port through which fluid may enter the column and a distal end with an outlet port through which fluid may exit the column; and

a bed of low nonspecific binding porous material within the column, the porous material having a second member capable of binding to the first member with an affinity constant of greater than about  $10^8 \text{ M}^{-1}$  immobilized on the surface thereof, wherein the pores of the porous material are of a size sufficient to allow the second member to enter into the pores and to allow entry of molecules greater than about 35 kD, but not so large as to allow collapse of the bed, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed.

2. A device for the separation of target particles indirectly bound to a first member from a mixture of target particles and non-target particles, comprising:

a column having a proximal end with an inlet port through which fluid may enter the column and a distal end with an outlet port through which fluid may exit the column; and

a bed of low nonspecific binding porous material within the column, the porous material having a second member capable of binding to the first member with an affinity constant of greater than about  $10^8 \text{ M}^{-1}$  immobilized on the surface thereof, the pores of the porous material being as large as possible without allowing collapse of the bed and of a size sufficient to allow the second member to enter into the pores, wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed.

3. A device for the separation of target particles indirectly bound to a first member from a mixture of target particles and non-target particles, comprising:

a column having a proximal end with an inlet port through which fluid may enter the column and a distal end with an outlet port through which fluid may exit the column;

a bed of low nonspecific binding porous polyacrylamide beads within the column, the beads having a second member capable of binding to the first member with an affinity constant of greater than about  $10^8 \text{ M}^{-1}$  immobilized onto the surface thereof, wherein the pores of the beads are of a size sufficient to allow entry of

molecules greater than about 60 kD into the pores, but not so large as to allow molecules of greater than about 300 kD into the pores, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and

a means for retaining the beads within the column.

4. A device for the separation of target particles directly or indirectly bound to a biotinylated ligand from a mixture of target particles and non-target particles, comprising:

a column having a proximal end with an inlet port through which fluid may enter the column and a distal end with an outlet port through which fluid may exit the column; and

a bed of low nonspecific binding porous material within the column, the porous material having a biotin adsorbing group immobilized onto the surface thereof, wherein the pores of the porous material are of a size sufficient to allow the biotin adsorbing group to enter into the pores, but not so large as to allow collapse of the bed, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed.

5. A device for the separation of target particles directly or indirectly bound to a biotinylated ligand from a mixture of target particles and non-target particles, comprising:

a column having a proximal end with an inlet port through which fluid may enter the column and a distal end with an outlet port through which fluid may exit the column;

a bed of low nonspecific binding porous polyacrylamide beads within the column, the beads having a biotin adsorbing group immobilized onto the surface thereof, wherein the pores of the beads are of a size sufficient to allow entry of molecules greater than about 60 kD into the pores, but not so large as to allow molecules of greater than about 300 kD into the pores, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and

a means for retaining the beads within the column.

6. A device for the separation of target particles indirectly bound to a first member from a mixture of target particles and non-target particles, comprising:

a column having a proximal end with an inlet port through which fluid may enter the column and a distal end with an outlet port through which fluid may exit the column; and

a bed of low nonspecific binding material within the column, the material having a second member capable of binding to the first member with an affinity constant of greater than about  $10^8 \text{ M}^{-1}$  immobilized on the surface thereof, wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and

a means for selectively agitating the material upon the application of an external force, such that bound target particles are fractionally released from the material, said means being located within the column.

7. A device for the separation of biotinylated target particles from a mixture of target and non-target particles, comprising:

a column having a proximal endcap with an inlet port through which fluid may enter the column and a distal endcap with an outlet port through which fluid may exit the column, wherein said endcaps are designed to retain a mesh;

a mesh retained in the upper and lower endcaps to retain beads within the column;

a bed of low nonspecific binding porous polyacrylamide beads within the column, the beads having a biotin adsorbing group immobilized onto the surface thereof, wherein the pores of the beads are of a size sufficient to allow entry of molecules greater than about 60 kD into the pores, but not so large as to allow molecules of greater than about 300 kD into the pores, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and

a magnetic impeller located within the column for agitating the porous polyacrylamide beads upon the application of an external force, such that bound target particles are released from the porous polyacrylamide beads.

8. The device of claim 7 wherein the beads have a particle size of about 170 microns to about 400 microns.

9. A device for the separation of target particles from a mixture of target particles and non-target particles, comprising:

a column having a proximal end with an inlet port through which fluid may enter the column and a distal end with an outlet port through which fluid may exit the column; and

a bed of low nonspecific binding porous material within the column, the porous material having a ligand capable of specifically binding to the target particles immobilized on the surface thereof, wherein the pores of the porous material are of a size sufficient to allow the ligand to enter into the pores and to allow entry of molecules greater than about 35 kD, but not so large as to allow collapse of the bed, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed.

10. A device for the separation of target particles from a mixture of target particles and non-target particles, comprising:

a column having a proximal end with an inlet port through which fluid may enter the column and a distal end with an outlet port through which fluid may exit the column; and

a bed of low nonspecific binding porous material within the column, the porous material having a ligand capable of specifically binding to the target particles immobilized on the surface thereof, the pores of the porous material being as large as possible without allowing collapse of the bed and of a size sufficient to allow the ligand to enter into the pores, wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed.

11. A device for the separation of target particles from a mixture of target particles and non-target particles, comprising:

a column having a proximal end with an inlet port through which fluid may enter the column and a distal end with an outlet port through which fluid may exit the column;

a bed of nonspecific binding porous polyacrylamide beads within the column, the beads having a ligand capable of specifically binding to the target particles immobilized onto the surface thereof, wherein the pores of the beads are of a size sufficient to allow entry of molecules greater than about 60 kD into the pores, but not so large as to allow molecules of greater than about 300 kD into the pores, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and

a means for retaining the beads within the column.

12. A device for the separation of target particles from a mixture of target particles and non-target particles, comprising:

a column having a proximal end with an inlet port through which fluid may enter the column and a distal end with an outlet port through which fluid may exit the column; and

a bed of low nonspecific binding material within the column, the material having a ligand capable of specifically binding to the target particles immobilized on the surface thereof, wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and

a means for selectively agitating the material upon the application of an external force, such that bound target particles are fractionally released from the porous material, said means being located within the column.

13. A sterile system for the separation of target cells from a mixture of target and non-target cells, comprising:

a sterile column, said column having a proximal end with an inlet port through which fluid enters the column and a distal end with an outlet port from which fluid exits the column;

a sterile packed bed of low nonspecific binding porous polyacrylamide beads located within the column, wherein the beads have avidin immobilized on the surface thereof, wherein the pores of the beads are of a size sufficient to allow the entry of molecules of greater than 60 kD into the pore, but not so large as to allow molecules of greater than about 300 kD into the pores, and wherein the interstitial spaces of the packed bed are of sufficient size to allow the cells to flow through the packed bed;

a sterile magnetic impeller located within the column;

a means for sterilely introducing a mixture of cells as well as sterile process fluids into the inlet port of the column;

a means for sterilely collecting cells and process fluids exiting from the outlet port of the column; and

an external source of electromagnetic force for regulating the rate of rotation of the impeller.

14. A method for separating target particles from a mixture of target particles and non-target particles, comprising:

incubating a mixture of target particles and non-target particles in the presence of a first member chemically linked to a ligand capable of specifically binding

to the target particles under conditions and for a time sufficient to allow binding of the ligand to the target particles;

passing the mixture of particles through a bed of low nonspecific binding porous material, the porous material having a second member capable of binding to the first member with an affinity constant of greater than about  $10^8 \text{ M}^{-1}$  immobilized on the surface thereof, wherein the pores of the porous material are of a size sufficient to allow the second member to enter into the pores and to allow entry of molecules greater than about 35 kD, but not so large as to allow collapse of the bed, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and

removing unbound non-target particles from the column.

15. A method for separating target particles from a mixture of target particles and non-target particles, comprising:

incubating a mixture of target particles and non-target particles in the presence of a first member chemically linked to a ligand capable of specifically binding to the target particles under conditions and for a time sufficient to allow binding of the ligand to the target particles;

passing the mixture of particles through a bed of low nonspecific binding porous material, the porous material having a second member capable of binding to the first member with an affinity constant of greater than about  $10^8 \text{ M}^{-1}$  immobilized on the surface thereof, the pores of the porous material being as large as possible without allowing collapse of the bed and of a size sufficient to allow the second member to enter into the pores, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and

removing unbound non-target particles from the column.

16. A method for separating target particles from a mixture of target particles and non-target particles, comprising:

incubating a mixture of target particles and non-target particles in the presence of a first member chemically linked to a ligand capable of specifically binding to the target particles under conditions and for a time sufficient to allow binding of the ligand to the target particles;

passing the mixture of particles through a bed of low nonspecific binding porous polyacrylamide beads, the beads having a second member capable of binding to the first member with an affinity constant of greater than about  $10^8 \text{ M}^{-1}$  immobilized onto the surface thereof, wherein the pores of the beads are of a size sufficient to allow



entry of molecules greater than about 60 kD into the pores, but not so large as to allow molecules of greater than about 300 kD into the pores, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and

removing unbound non-target particles from the column.

17. A method for separating target particles from a mixture of target particles and non-target particles, comprising:

incubating the mixture of target particles and non-target particles in the presence of a first ligand capable of specifically binding to the target particles under conditions and for a time sufficient to allow binding of the first ligand to the target particles;

incubating the mixture of target particles and non-target particles in the presence of a first member chemically linked to a second ligand capable of binding to the first ligand under conditions and for a time sufficient to allow binding of the second ligand to the first ligand on the target particles;

passing the mixture of particles through a bed of low nonspecific binding porous material, the porous material having a second member capable of binding to the first member with an affinity constant of greater than about  $10^8 \text{ M}^{-1}$  immobilized on the surface thereof, wherein the pores of the porous material are of a size sufficient to allow the second member to enter into the pores and to allow entry of molecules greater than about 35 kD, but not so large as to allow collapse of the bed, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and

removing unbound non-target particles from the column.

18. A method for separating target particles from a mixture of target particles and non-target particles, comprising:

incubating the mixture of target particles and non-target particles in the presence of a first ligand capable of specifically binding to the target particles under conditions and for a time sufficient to allow binding of the first ligand to the target particles;

incubating the mixture of target particles and non-target particles in the presence of a first member chemically linked to a second ligand capable of binding to the first ligand under conditions and for a time sufficient to allow binding of the second ligand to the first ligand on the target particles;

passing the mixture of particles through a bed of low nonspecific binding porous material, the porous material having a second member capable of binding to the first member with an affinity constant of greater than about  $10^8 \text{ M}^{-1}$  immobilized on the surface thereof, the pores of the porous material being as large as possible without allowing collapse of the bed and of a size sufficient to allow the second member to enter into the pores, and wherein the interstitial spaces of the bed are of sufficient size to allow the particles to flow through the bed; and

removing unbound non-target particles from the column.

19. A method for separating target particles from a mixture of target particles and non-target particles, comprising:

incubating the mixture of target particles and non-target particles in the presence of a first ligand capable of specifically binding to the target particles under conditions and for a time sufficient to allow binding of the first ligand to the target particles;

incubating the mixture of target particles and non-target particles in the presence of a first member chemically linked to a second ligand capable of binding to the first ligand under conditions and for a time sufficient to allow binding of the second ligand to the first ligand on the target particles;

passing the mixture of particles through a bed of low nonspecific binding porous polyacrylamide beads, the beads having a second member capable of binding to the first member with an affinity constant of greater than about  $10^8 \text{ M}^{-1}$  immobilized onto the surface thereof, wherein the pores of the beads are of a size sufficient to allow entry of molecules greater than about 60 kD into the pores, but not so large as to allow molecules of greater than about 300 kD into the pores, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and

removing unbound non-target particles from the column.

20. A method for separating target particles from a mixture of target particles and non-target particles, comprising:

passing the mixture of particles through a bed of low nonspecific binding porous material, the porous material having a ligand capable of specifically binding to the target particles immobilized on the surface thereof, wherein the pores of the porous material are of a size sufficient to allow the ligand to enter into the pores and to allow entry of molecules greater than about 35 kD, but not so large as to allow collapse of the

bed, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and

removing unbound non-target particles from the column.

21. A method for separating target particles from a mixture of target particles and non-target particles, comprising:

passing the mixture of particles through a bed of low nonspecific binding porous material, the porous material having a ligand capable of specifically binding to the target particles immobilized on the surface thereof, the pores of the porous material being as large as possible without allowing collapse of the bed and of a size sufficient to allow the ligand to enter into the pores, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and

removing unbound non-target particles from the column.

22. A method for separating target particles from a mixture of target particles and non-target particles, comprising:

passing the mixture of particles through a bed of low nonspecific binding porous polyacrylamide beads, the beads having a ligand capable of specifically binding to the target particles immobilized onto the surface thereof, wherein the pores of the beads are of a size sufficient to allow entry of molecules greater than about 60 kD into the pores, but not so large as to allow molecules of greater than about 300 kD into the pores, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed; and

removing unbound non-target particles from the column.

23. A method according to any one of claims 14 through 22, further comprising, subsequent to the step of removing, releasing the bound target particles from the porous material.

24. A method according to claim 23 wherein the step of releasing comprises fractionally releasing the bound target particles.

25. A method according to any one of claims 14 through 22, further comprising passing the mixture of particles through a prefilter prior to passing the mixture through the bed of porous material, such that debris, particulates and aggregates large enough to block fluid flow through the interstitial spaces are removed.

26. A method according to any one of claims 14 through 22, further comprising, subsequent to the step of removing, collecting the unbound non-target particles.

27. A method according to any one of claims 14 through 19 wherein the first member-second member binding pair is selected from the group consisting of biotin-avidin, biotin-streptavidin, biocytin-avidin, biocytin-streptavidin, methotrexate-dihydrofolate reductase, 5-fluorouracil-thymidylate synthetase, riboflavin-riboflavin binding protein, avidin-biotin, streptavidin-biotin, avidin-biocytin, streptavidin-biocytin, dihydrofolate reductase-methotrexate, thymidylate synthetase-5 fluorouracil, and riboflavin binding protein-riboflavin.

28. A method according to claim 17, 18, or 19 wherein the first ligand is selected from the group consisting of antibodies, lectin, substrate molecules, enzyme inhibitors, binding proteins and proteins.

29. A method according to any one of claims 14 through 22 wherein the bed is a packed bed.

30. A method according to any one of claims 14, 15, 16, 20, 21 or 22 wherein the ligand is selected from the group consisting of antibodies, antibody fragments, anti-particle surface receptor molecules, and transferrin.

31. A method according to any one of claims 14, 15, or 16 wherein the mixture of particles is incubated with an excess amount of a first member chemically linked to a ligand.

32. A method according to any one of claims 17, 18, or 19 wherein the mixture of particles is incubated with an excess amount of the first ligand.

33. A method according to any one of claims 17, 18, or 19 wherein the mixture of particles is incubated with an excess amount of a first member chemically linked to a second ligand.

34. A method according to any one of claims 14, 15, or 16, further comprising removing unbound first member chemically linked to a ligand from the mixture prior to passing the mixture through the bed.

35. A method according to any one of claims 17, 18, or 19, further comprising removing unbound first ligand prior to incubating in the presence of the first member chemically linked to a second ligand.

36. A method according to any one of claims 17, 18, or 19, further comprising removing unbound first member chemically linked to a second ligand prior to passing the mixture through the bed.

37. A method according to any one of claims 14 through 22 wherein the particles are selected from the group consisting of viruses, bacteria, fungi, parasites, and cells.

38. A method according to any one of claims 14 through 22 wherein the steps are performed in a sterile manner, such that external organisms do not enter into the mixture.

39. A device according to any one of claims 1, 2, 4, 9, or 10 wherein the porous material is selected from the group consisting of porous hollow fibers, porous solid fibers, porous beads, and porous magnetic beads.

40. A device according to any one of claims 1, 2, 4, 9, or 10 wherein the device further comprises a means for agitating the porous material, located within the column, upon the application of an external force, such that bound target particles are released from the porous material.

41. A device according to claim 40 wherein the means for agitating the porous material is selected from the group consisting of magnetic impellers, magnetic beads, weights, magnetic weights, and buoyant floats.

42. A device according to claim 40 wherein the external force is selected from the group consisting of electromagnetic forces, gravitational forces, and mechanical forces.

43. A device according to either one of claims 3 or 5 wherein the device further comprises a means, located within the column, for agitating the porous

polyacrylamide beads upon the application of an external force, such that bound target particles are released from the porous polyacrylamide beads.

44. A device according to claim 43 wherein the means for agitating the porous polyacrylamide beads is selected from the group consisting of impellers, magnetic beads, weights, magnetic weights, and buoyant floats.

45. A device according to claim 43 wherein the external force is selected from the group consisting of electromagnetic forces, gravitational forces, and mechanical forces.

46. A device according to claim 6, 7 or 12 wherein the external force is selected from the group consisting of electromagnetic forces, gravitational forces and mechanical forces.

47. A device according to claim 6 or 12 wherein the means for selectively agitating the material is selected from the group consisting of impellers, magnetic beads, weights, magnetic weights and buoyant floats.

48. A device according to claim 39 wherein the external force is provided by a magnetic stir plate, magnetic coils, a sonicator, a rocking plate, or a vortex.

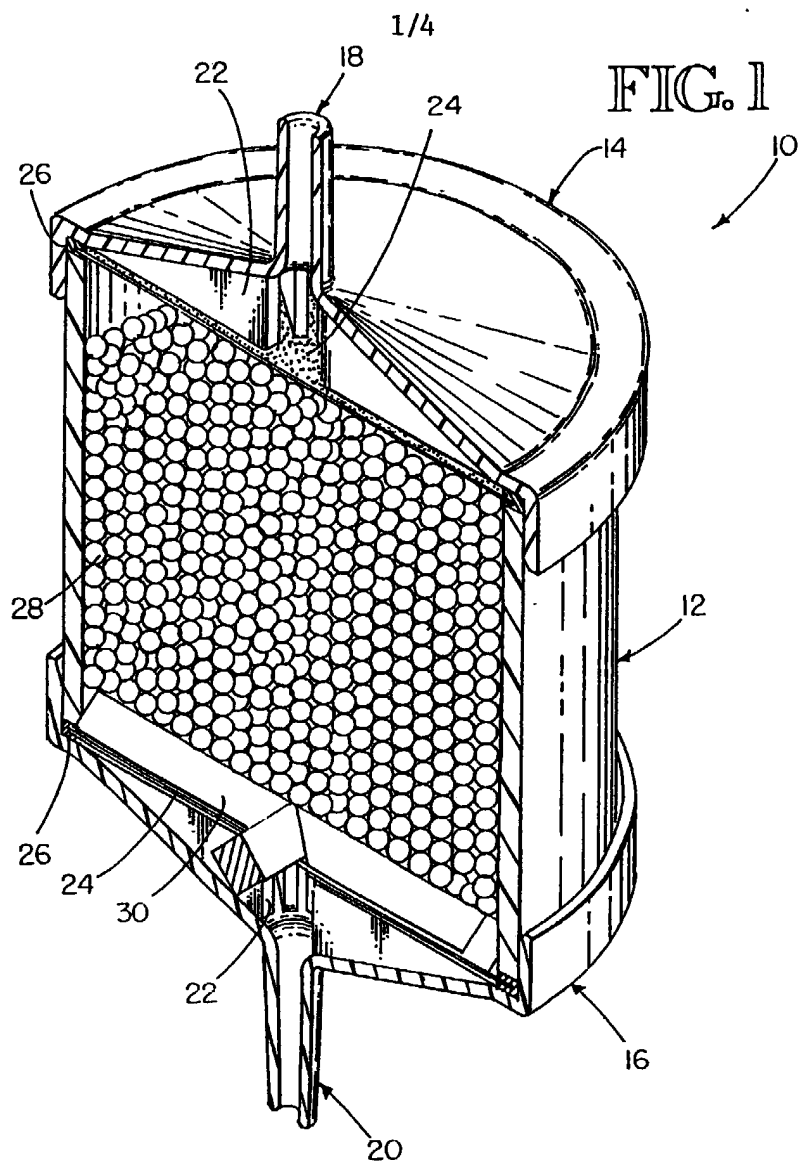
49. A device according to claim 43 wherein the external force is provided by a magnetic stir plate, magnetic coils, a sonicator, a rocking plate, or a vortex.

50. A device according to any one of claims 1, 2, or 3 wherein the second member is selected from the group consisting of biotin, biocytin, avidin, streptavidin, methotrexate, dihydrofolate reductase, 5-fluorouracil, thymidylate synthetase, riboflavin, and riboflavin binding proteins.

51. A device according to claim 4 or 5 wherein the biotin adsorbing group is selected from the group consisting of avidin, avidin analogs, porin, antibodies and combinations thereof.

52. A device according to claim 1, 2, 3, 6, 7, 9, 10, 12, or 13 wherein said bed contains benzoic acid.

53. A device according to claim 1, 2, 3, 6, 7, 9, 10, 12, or 13, wherein said bed contains a compound selected from the group consisting of sodium azide, benzoic acid, sorbate, and dehydroacetic acid.





2/4

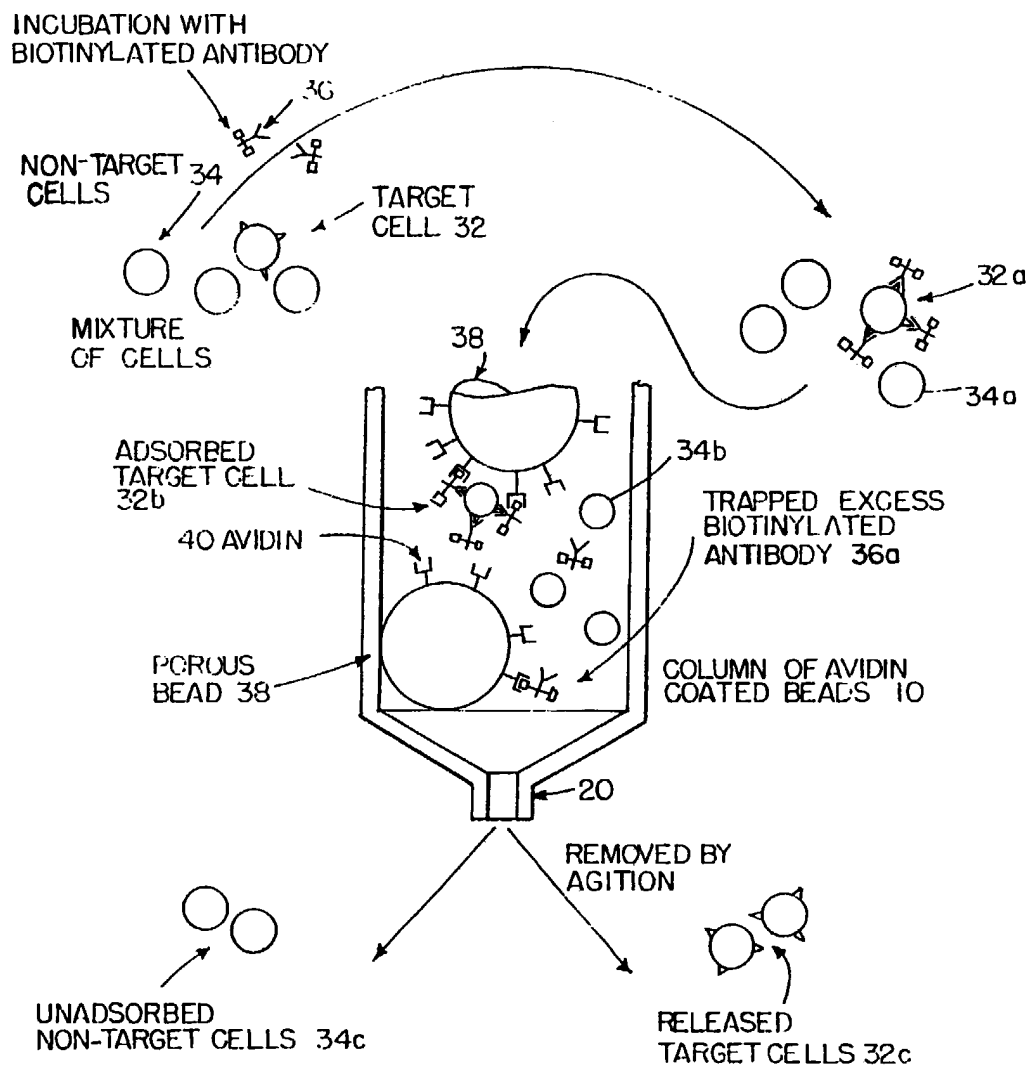


FIG. 2

3/4

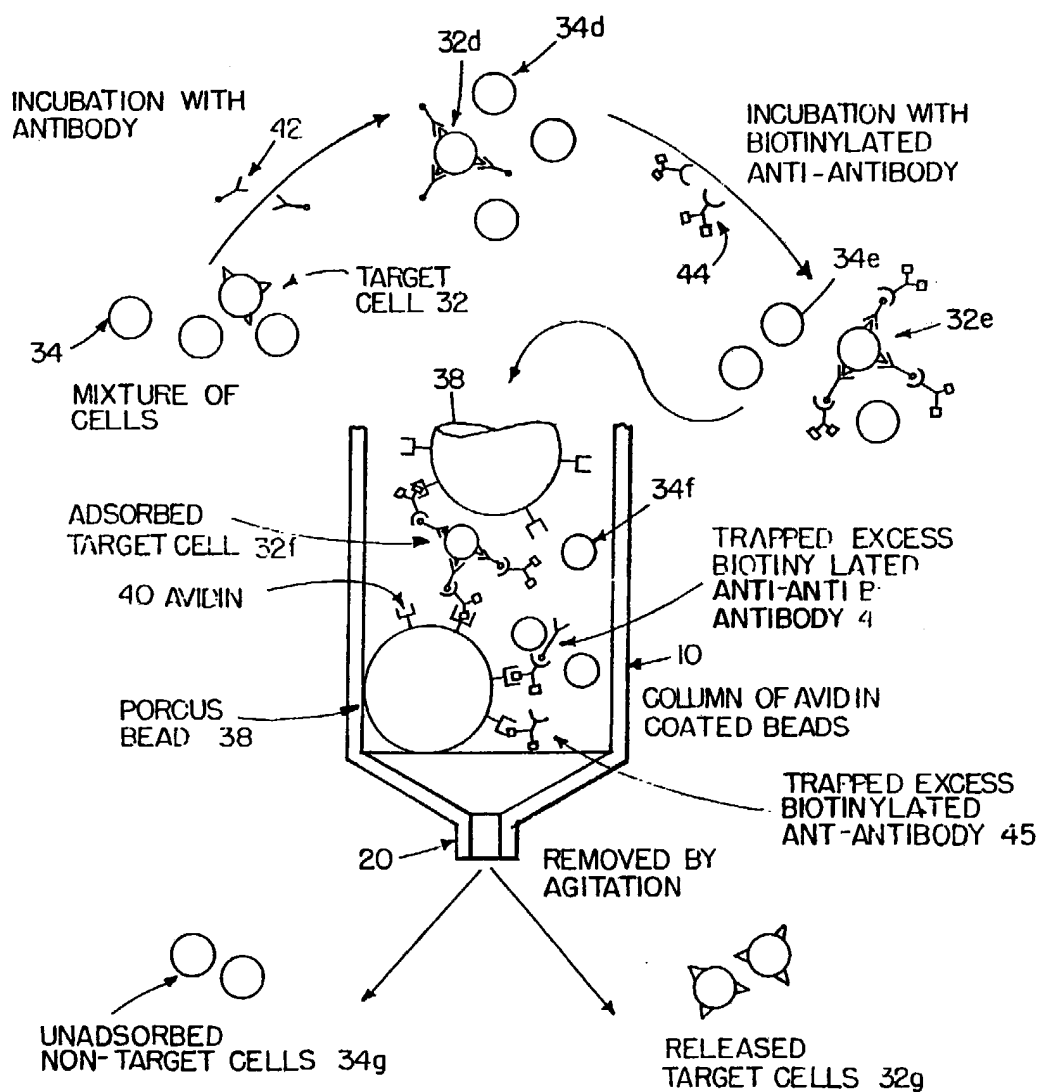


FIG. 3

4/4

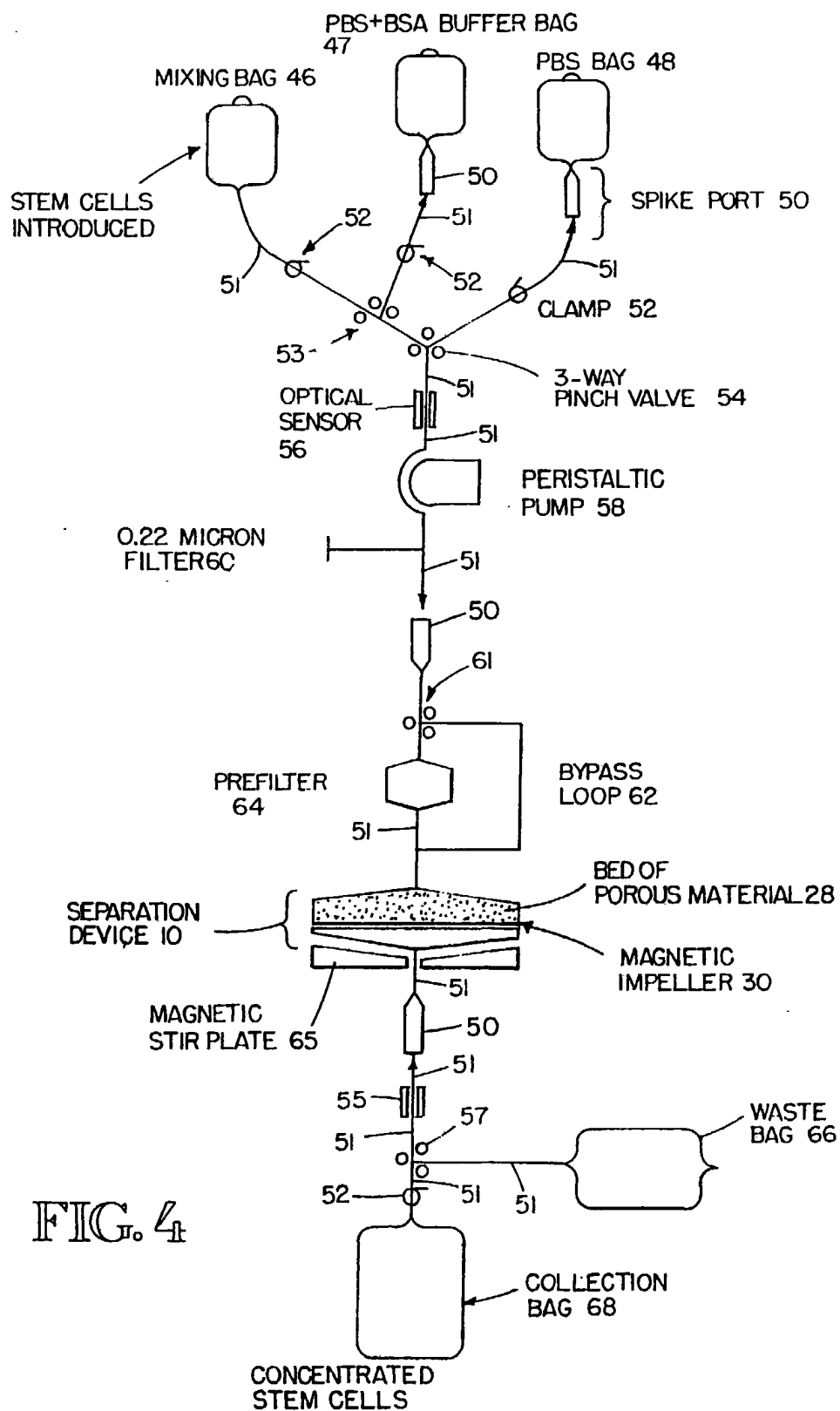


FIG. 4

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 91/02780

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.C1.5                      B 01 D 15/08                      C 12 Q 1/24 // G 01 N 33/569		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.C1.5	G 01 N                      C 12 Q                      B 01 D	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>o</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	WO,A,8704628 (FRED HUTCHINSON CANCER RESEARCH CENTER) 13 August 1987, see whole document (cited in the application) ---	1-53
X	JOURNAL OF IMMUNOLOGICAL METHODS, vol. 91, 1986, pages 11-19, Elsevier Science Publishers B.V., R.J. BERENSON et al.: "Positive selection of viable cell populations using avidin-biotin immunoadsorption", see whole article ---	1-53
A	EP,A,0254117 (FUJI PHOTO FILM CO., LTD) 27 January 1988 ---	
A	EP,A,0339787 (CAMBRIDGE BIOMEDICAL LTD) 2 November 1989 -----	
<div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p><sup>o</sup> Special categories of cited documents : <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
21-08-1991	27. 09. 91	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	miss T. MORTENSEN	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9102780

SA 47595

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 18/09/91  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8704628	13-08-87	EP-A- 0260280 JP-T- 63502965	23-03-88 02-11-88
EP-A- 0254117	27-01-88	JP-A- 63019559 US-A- 4868131	27-01-88 19-09-89
EP-A- 0339787	02-11-89	GB-A- 2218200	08-11-89

EPO FORM P473

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82